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# Reticulospinal plasticity after cervical spinal cord injury in the rat involves withdrawal of projections below the injury

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ABSTRACT

Restoring voluntary fine motor control of the arm and hand is one of the main goals following cervical spinal cord injury (SCI). Although the functional improvement achievable with rehabilitative training in rat models is frequently accompanied by corticospinal tract (CST) plasticity, CST rewiring alone seems insufficient to account for the observed recovery. Recent investigations in animal models of SCI have suggested that the reticulospinal tract (RtST) might contribute to mediating improved motor performance of the forelimb. Here we investigate whether the spared RtST can compensate for the loss of CST input and whether RtST projections rearrange in response to cervical SCI. Animals underwent unilateral ablation of the dorsal CST and rubrospinal tract at spinal level C4, while the ventral RtST projections were spared. At the end of the six-week recovery period, injured animals had made significant improvements in single pellet reaching. This was not accompanied by increased sprouting of the injured CST above the injury compared to uninjured control animals. Injury-induced changes in RtST fiber density within the gray matter, as well as in the number of RtST collaterals entering the gray matter or crossing the cord midline were minor above the injury. However, all analyses directly below the injured spinal level consistently point to a significant decrease of RtST projections. The mechanism and the functional relevance behind this new finding warrant further study. Our results also suggest that mechanisms other than anatomical plasticity, such as plastic changes on a cellular level, might be responsible for the observed spontaneous recovery.



## Introduction

Cervical spinal cord injury (SCI) results in the disruption of descending pathways important for motor control of the extremities. One such pathway, which has been the focus of intense research efforts to reorganize the injured spinal cord (Oudega and Perez, 2012), is the corticospinal tract (CST), the main pathway to contribute to fine motor control of the distal arm and hand in humans (Schieber, 2007). In rats, the injured CST has repeatedly been reported to sprout at cervical level in response to thoracic SCI, thereby building novel connections with lesion-bridging relay neurons that may then transmit the signal to denervated neurons below the injury (Bareyre et al., 2004; Fouad et al., 2001). In contrast, CST sprouting rostral to a cervical lesion was only reported when rehabilitative training was added during the recovery period (Girgis et al., 2007). Yet, recent investigations have led to the conclusion that corticospinal plasticity alone might not suffice to mediate the observed degree of recovery following training in forelimb motor tasks (Krajacic et al., 2010).

A tract well suited to contribute to the recovery of reaching in rats is the rubrospinal tract (RST; Kanagal and Muir, 2009; Morris et al., 2011). Interestingly, lesions that involve both the CST and the RST still allow substantial spontaneous and training induced recovery (Krajacic et al., 2010). As a consequence, the reticulospinal tract (RtST), which projects mainly in the ventrolateral funiculus (Martin et al., 1985; Waldron and Gwyn, 1969; Zemlan et al., 1984), has gained attention as another potential contributor to the recovery of forelimb function after CST lesions in cats and primates (Pettersson et al., 2007). Reticular neurons receive cortical input and have been shown to project to spinal motoneurons that control a variety of upper extremity muscles (Davidson and Buford, 2006; Riddle et al., 2009). RtST axons commonly regenerate more readily than CST axons (Vavrek et al., 2007; Xu et al., 1995) and exhibit the ability to sprout and rewire following SCI (Ballermann and Fouad, 2006). Therefore, the RtST might prove useful in efforts to improve voluntary motor control of the upper limb following ablation of the dorsal CST. In addition, the RtST is mostly spared by dorso-lateral lesions of the spinal cord (which disrupt the dorsal CST), since its axons project within a large portion of the ventral and ventrolateral white matter in the spinal cord (Martin et al., 1985; Waldron and Gwyn, 1969; Zemlan et al., 1984). In an effort to promote new

*Abbreviations:* AAV, Adeno-associated viral vectors; BDNF, brain-derived neurotrophic factor; CST, corticospinal tract; DAB, Diaminobenzidine; GFP, green-fluorescent protein; NT-3, Neurotrophin 3; p.i, post-injury; RST, rubrospinal tract; RtST, reticulospinal tract; SCI, spinal cord injury.

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connections between the CST and RtST in the brainstem in the present study, we injected adeno-associated viral vectors (AAV) expressing brain-derived neurotrophic factor (BDNF; to promote collateral growth) and neurotrophin-3 (NT-3; to serve as a chemo-attractant to direct CST sprouting towards the reticular formation). GFP-expressing vectors were used as a control. Viral vector mediated neurotrophin treatment did not promote observable plasticity or recovery. Yet, limited spontaneous recovery irrespective of AAV treatment was evident, which has been suggested to involve RtST function (Krajacic et al., 2010; Pettersson et al., 2007). Whether this potential contribution of the RtST to recovery also includes re-organization of the tract's projection pattern is currently unknown. To investigate this, RtST projection patterns were analyzed above and below the C4 injury level using three different outcome measures. AAV-treated, injured animals were pooled and compared to AAV-treated, uninjured controls to examine solely the effect of injury on RtST plasticity.

## Materials and methods

### *Animals and experimental groups*

A total of 26 female Lewis rats (Charles River Laboratories, Canada) weighing 180 g–200 g were group housed at a 12 h:12 h light/dark cycle. Twenty-one animals received a unilateral incomplete cervical SCI. In an effort to promote plasticity, these animals received the following injections of adeno-associated viral vectors (AAV) of serotype 2: AAV expressing brain-derived neurotrophic factor (BDNF) in the motor cortex and neurotrophin-3 (NT-3) in the reticular formation ($n = 7$), AAV expressing the non-pharmacologically active green-fluorescent protein (GFP) in the motor cortex and NT-3 in the reticular formation ($n = 7$) or GFP-expressing AAV into both locations ($n = 7$). Five control animals remained unlesioned but received BDNF-expressing AAV into the motor cortex and NT-3 expressing AAV into the reticular formation to provide a close control condition for detecting an injury-induced effect. Three animals remained unlesioned and did not receive AAV injections. Some animals had to be excluded from individual histological analyses because data could not be obtained from a sufficient number of sections meeting the necessary quality standards (e.g., tears or folding of tissue, which would produce significant artifacts in densitometry measures). The number of animals included in each analysis is indicated in brackets in the Results section. Animals were fed ad libitum except for the day preceding single pellet reaching sessions when food pellets were reduced to 8 g/rat. Weights were closely monitored to ensure stable body weight over time. All procedures involving animals were approved by the Health Sciences Animal Care and Use Committee at the University of Alberta.

### *Spinal cord injury and AAV injection*

Animals in the injured groups received a dorso-lateral quadrant spinal lesion unilateral to their preferred paw (as established during reaching training). Rats were anesthetized by a subcutaneous injection of Fentanyl (0.2 mg/kg, Hypnorm, Janssen Pharmaceutics, Beerse, Belgium) mixed with Midazolam (4 mg/kg, Versed, Sabex, Boucherville, QC, Canada). The surgical area was shaved and disinfected and the animal mounted into a stereotactic frame (Kopf Instruments, Tujunga, CA, USA). Throughout the surgery, body temperature was maintained at 37 °C with a heating blanket. Following a skin incision and dissection of muscle, the spinal cord between C3 and C4 was exposed with a laminectomy of half a vertebral segment (C3). A custom-made microblade was lowered 1 mm into the spinal cord at the midline, then moved lateral. Muscle layers were sutured and the skin was closed with staples.

In injured as well as in 5 uninjured animals, the brain was accessed via two drill holes to allow injection of AAV vectors. The forelimb motor cortex contralateral to lesion was targeted at coordinates 1.5 mm anterior and 1.5 mm lateral to bregma, 1.5 mm below the dura. The gigantocellular nucleus of the reticular formation ipsilateral to lesion was targeted at 2.8 mm caudal and 0.8 mm lateral to bregma, at a depth of 9.2 mm. One microliter of the respective AAV in solution was slowly pressure injected into each location at 13–25 psi using a custom-made glass electrode connected to a picospritzer. The incision was then closed with stitches. Post-operative hydration was ensured by s.c. injection of 4 ml saline and pain was managed by s.c. injections of buprenorphine (0.05 mg/kg, Temgesic, Schering-Plough, QC, Canada). Animals were kept on a heating blanket until fully awake.

### *Single pellet reaching*

Before lesion surgery, all animals assigned to injured groups were trained to reach through a slot (1.5 cm wide) in a Plexiglas box ($15 \times 36 \times 30$ cm) to grasp sugar pellets offered to them in a small indentation on a tray (pellet 2 cm away from front wall at a height of 3 cm above the elevated grid floor). In addition, the rats were taught to go back to the other end of the box before the next pellet was offered. Success rates per session were calculated as the percentage of pellets successfully grasped and eaten out of 20 pellets offered. Starting at day seven post-injury (p.i.), all animals were tested in reaching twice per week. Performance scores before the injury, at the beginning of reaching testing p.i. and at the end of the recovery period were determined by selecting the best performance for each animal out of three consecutive sessions.

### *Horizontal ladder*

Rats were videotaped while crossing an elevated horizontal ladder with rungs (1.5 mm in diameter) randomly spaced between 2 cm and 5 cm apart (Bolton et al., 2006). Before lesion surgery, animals were familiarized with the task and then three ladder crossings were videotaped with a JVC digital camera and analyzed frame-by-frame on a computer screen for baseline scores. Final testing was done the same way at the end of the recovery period. An error made by the preferred forepaw was defined as a fall or a deep slip with either the rat losing balance or the paw dropping underneath the rung level up to the point of the carpal/tarsal joint. Error rate was calculated by averaging the score of three ladder crossings and expressed as percentage of erroneous steps made by the preferred/injured paw out of the total number of steps taken to cross the ladder.

### *Cylinder test*

At the end of the recovery period, rats were filmed with a JVC digital camera as they explored the walls of a clear Plexiglas cylinder (24 cm high, 19 cm inner diameter; Schallert et al., 2000). Spontaneous usage of either forepaw was analyzed frame-by-frame on a computer screen by counting how many times either paw touches the wall of the cylinder during ten rearings. The sum of paw touches was used to calculate to what percentage the ipsilateral paw was engaged in exploration.

### *Tracing*

After a recovery time of 6 weeks, the forelimb motor cortex contralateral to the spinal lesion was injected at 3 locations with 1 μl of Alexa Fluor 488 (10%, Molecular Probes, Eugene, OR, USA) at a depth of 1.5 mm under isoflurane anesthesia. Neurons in the gigantocellular division of the reticular formation in the brainstem were traced by injecting 1 μl of Fluororuby (10%, Molecular Probes, Eugene, OR, USA) at coordinates 2.8 mm posterior and 0.8 mm lateral to lambda, 9.2 mm below the dura, on the side ipsilateral to the spinal lesion. Fluororuby administration was performed by pressure injection with a picospritzer at pulses of 15 ms duration at 13–25 psi. The incision was closed with stitches and post-operative care was as